

AD-A263 177



MENTATION PAGE

Form Approved
OMB No. 0704-0188

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REPORT DATE		3. REPORT TYPE AND DATES COVERED	
4. TITLE AND SUBTITLE Effects of chronic stress on anterior pituitary and brain corticotropin-releasing factor receptors.		5. FUNDING NUMBERS 611102A 3M161102BS S15CD	
6. AUTHOR(S) S.M. Anderson, G.J. Kant, E.B. DeSouza			
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Department of Medical Neurosciences Division of Neuropsychiatry Walter Reed Army Institute of Research Washington, D.C. 20307-5100		8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES) US Army Medical Research and Development Command Fort Detrick, Frederick MD 21701		10. SPONSORING/MONITORING AGENCY REPORT NUMBER	
11. SUPPLEMENTARY NOTES			
12a. DISTRIBUTION/AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited,			
12b. DISTRIBUTION CODE			
13. ABSTRACT (Maximum 200 words) [¹²⁵ I]Corticotropin-releasing factor ([¹²⁵ I]CRF) binding was measured in membrane homogenates from anterior pituitary and 8 brain regions from control and chronically stressed rats. The stressor consisted of 3 or 14 days of around-the-clock intermittent footshock avoidance/escape (ITI = 5 min). Plasma corticosterone levels were almost doubled in stressed rats following 3 days of chronic stress and remained significantly elevated in rats stressed for 14 days vs. controls. [¹²⁵ I]CRF binding was decreased in anterior pituitary and frontal cortex following three days of chronic stress; binding affinity of anterior pituitary membranes was not different between control and stressed animals. [¹²⁵ I]CRF binding was similar in control and 3 day stressed animals in the other brain areas examined. After 14 days of chronic stress, hypothalamic [¹²⁵ I]CRF binding was decreased in stressed rats as compared to control animals but no other differences were seen.			
14. SUBJECT TERMS Stress, corticotropin-releasing factor receptors, CRF, CRH, corticotropin-releasing hormone, neuroendocrine factor receptors		15. NUMBER OF PAGES	
		16. PRICE CODE	
17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT

NSN 7540-01-280-5500

ANNEX C

Standard Form 298 (Rev. 2-89)
Prescribed by ANSI Std. Z39-18
298-102

Effects of Chronic Stress on Anterior Pituitary and Brain Corticotropin-Releasing Factor Receptors

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Received 30 October 1989

ANDERSON, S. M., G. J. KANT AND E. B. DE SOUZA. *Effects of chronic stress on anterior pituitary and brain corticotropin-releasing factor receptors*. PHARMACOL BIOCHEM BEHAV 44(4) 755-761, 1993. — [¹²⁵I]Corticotropin-releasing factor binding was measured in membrane homogenates prepared from the anterior pituitary, frontal cortex, motor cortex, somatosensory cortex, mesolimbic area (olfactory tubercle and nucleus accumbens), caudate putamen, hypothalamus, midbrain, and cerebellum from control and chronically stressed rats. The stressor consisted of 3 or 14 days of around-the-clock intermittent foot-shock (approximately one trial per 5-min frequency) that could be avoided or escaped on 90% of the trials presented by pulling a ceiling chain. Plasma corticosterone levels were almost doubled in stressed rats following 3 days of chronic stress and remained significantly elevated in rats stressed for 14 days as compared to controls. Plasma corticotropin levels were similar in controls and stressed animals in both the 3- and 14-day experiments. [¹²⁵I]Corticotropin-releasing factor binding was decreased in anterior pituitary and frontal cortex following 3 days of chronic stress; binding affinity of anterior pituitary membranes was not different between control and stressed animals. [¹²⁵I]Corticotropin-releasing factor binding was similar in control and 3-day-stressed animals in the other brain areas examined. After 14 days of chronic stress, hypothalamic [¹²⁵I]corticotropin-releasing factor binding was decreased in stressed rats as compared to control animals but no other differences were seen. The decrease in the apparent number of anterior pituitary corticotropin-releasing factor receptors following 3 days of stress may be due, in part, to increased plasma corticosterone levels and/or increased corticotropin-releasing factor secretion during that time. The downregulation of frontal cortex and hypothalamic corticotropin-releasing factor receptors, however, is more likely to be primarily in response to sustained stress-induced corticotropin-releasing factor release at those sites.

Stress Corticotropin-releasing factor CRF receptors Neurohumor receptors Anterior pituitary gland

ONE of the initial neuroendocrine responses to stress is an increase in plasma corticotropin (ACTH) and other proopiomelanocortin (POMC)-derived peptides (8,32,45). ACTH, released from corticotrophs in the pituitary gland, stimulates secretion of glucocorticoids from the adrenal glands. Purification and isolation of the 41-residue peptide corticotropin-releasing factor (CRF) by Vale and coworkers and subsequent experimentation using synthetic CRF identified that peptide as the primary regulator of the pituitary secretion of ACTH (18). CRF is synthesized by cell bodies in the paraventricular nucleus of the hypothalamus, transported to the median emi-

nence, then released into the portal vessels, and acts at membrane bound receptors in the anterior pituitary via adenylate cyclase to cause secretion of ACTH (18,45).

In addition to its neuroendocrine role as the dominant initiator of ACTH secretion in response to stress, identification of CRF immunoreactive neurons (34,41,48) and the presence of CRF receptors in the CNS (11,13,52) suggest a neurotransmitter/neuromodulator function for CRF. The proposal is reinforced by recent evidence of CRF's ability to stimulate autonomic function, alter electrophysiological activity, and influence a variety of behaviors (18,45). Further, altered CRF

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content (3,5,14,19,23,29,39,49) and density of brain CRF receptors (15,16) have been demonstrated in some neuropsychiatric and neurodegenerative disorders.

CRF immunoreactivity in the periventricular and anterior hypothalamic nuclei (9) and paraventricular nucleus (46) has been shown to increase in response to chronic stress. As in many other receptor systems, CRF receptor downregulation has been demonstrated in pituitary after both *in vitro* and *in vivo* chronic exposure to its endogenous agonist (43,51). Downregulation and desensitization of anterior pituitary CRF receptors by chronic infusion of CRF in intact animals was associated with a decrease in the ability of CRF to stimulate cyclic adenosine monophosphate (cAMP) formation and ACTH release in isolated pituitary cells prepared from rats receiving CRF infusions (51). Such receptor regulation may represent an adaptive response to excessive hormone secretion during periods of stress.

We have been investigating neuroendocrine and neurochemical effects of stress in rats using a variety of acute and chronic stress paradigms (2,25-28). Here, we present experiments showing a downregulation of CRF receptors in membranes from the anterior pituitary, frontal cortex, and hypothalamus in rats exposed to chronic stress.

METHOD

Animals

Male Sprague-Dawley rats (200-225 g) were used in these experiments. Rats were purchased from Zivic-Miller and housed in our animal housing area for a minimum of 2 weeks prior to the beginning of each experiment. Animals were individually caged during this holding period with food (Ziegler rat diet) and water freely available. Lights were on from 0600-1800 h daily.

Stress Exposure

Each experiment was conducted using eight rats. Four rats served as controls and four as the experimental stress group. All rats were housed 24 h per day in standard operant cages equipped with a lever, water bottle, stimulus lights, a sonalert (to deliver a tone warning), parallel metal floor bars, and a centrally located ceiling chain. Each cage was placed inside a sound-attenuating box enclosure equipped with a houselight and fan. Houselights within the enclosure were on from 0600-1800 h and off from 1800-0600 daily. Food pellets were delivered following each lever press. Rats were allowed 4 days to habituate to the new environment and learn to lever press for food. After 4 days in the environment, four rats were trained to pull the ceiling chain to terminate foot-shock delivered by the experimenter. After this was learned (in general 15-30 min of training sufficed), shock trials were regulated by a PDP11 computer programmed in SKED. Until 35 shock trials had been successfully escaped, trials were presented every minute. After this initial period, shock trials were presented on a variable schedule on the average of one per 5 min around the clock. Each trial consisted of a 5-s warning light, followed by 5 s of a warning tone, followed by 5 s each of five increasing shock levels (0.16, 0.32, 0.65, 1.3, and 2.6 mA). For the first 24 h in this environment, all shock trials could be avoided by pulling the chain during the warning light or tone or escaped at any shock level by pulling the chain. For rats that successfully escaped more than 75% of the trials presented, 10% of the trials during the next 24 h were made nonescapable, that is, pulling the chain had no effect on these trials. Rat perfor-

mance was reviewed at least daily and rats that did not maintain a 75% avoid/escape rate of escapable trials were placed back on the original schedule with all trials being escapable. Twenty consecutive nonescapes shut off the shock presentations completely to avoid any possibility of excessive trauma. Controls lived in identical cages but were never exposed to foot-shock. In one series of experiments, rats lived in this environment for 3 days of intermittent shock. Rats were exposed to 14 days of intermittent foot-shock using this paradigm in a second series of experiments. The 3- and 14-day behavioral experiments were not done contemporaneously and were conducted over several months. All lever presses for food and chain pulls were recorded and stored by the PDP11 computer. To obtain a reasonable sample size, behavioral and biochemical data were gathered on seven separate occasions in similar test runs that were conducted over a period of several months. The data presented here are values pooled from those separate experimental runs.

Experimental Procedures

After 3 or 14 days of stress trials, rats were killed by decapitation. All animals were killed between 0900 and 1100 h to minimize the effects of circadian variation (27). Trunk blood samples were collected in heparinized beakers, transferred to polypropylene test tubes, and aprotinin (Sigma A-6012, Sigma Chemical Co., St. Louis, MO), a peptidase inhibitor, was added (0.55 TIU/ml). The blood samples were centrifuged at 4°C and two aliquots of plasma were stored at -70°C until assayed for corticosterone and ACTH. Anterior pituitaries were removed, weighed, and immediately frozen on dry ice. Brains were removed rapidly and chilled in ice-cold saline. Olfactory bulbs were removed, weighed, and frozen on dry ice. Two- and 3-mm coronal brain slices were cut using a Plexiglas block as described by Jacobowitz (24). The frontal cortex, the mesolimbic area (olfactory tubercle and nucleus accumbens), the caudate putamen, sections of motor and somatosensory cortex, the hypothalamus, a section of midbrain containing the locus coeruleus, and the cerebellum were dissected from those slices using Paxinos and Watson's atlas of the rat brain as the reference (42). The brain regions were weighed and frozen on dry ice. The anterior pituitary glands and all brain regions were stored at -70°C for receptor assay at a later date.

Hormone Assays

Corticosterone was measured by radioimmunoassay using an antibody produced in rabbits by our laboratory against corticosterone-21-hemisuccinate: bovine serum albumin (BSA). Somogyi reagents were used to separate free from bound ligand, corticosterone [$1,2\text{-}^3\text{H}(\text{N})$] (New England Nuclear Corp., Newton, MA, $\text{sa} = 50 \text{ Ci/mmol}$). Assay sensitivity was 0.6 $\mu\text{g/dl}$. The intraassay and interassay coefficients of variation were 6 and 12% respectively (36).

ACTH was measured using a radioimmunoassay kit (INCSTAR Corp., Stillwater, MN). One hundred microliters of plasma, rabbit anti-ACTH serum, and [^{125}I]ACTH were incubated in polypropylene test tubes (12 \times 75 mm) overnight at 4°C. Samples were then incubated for 30 min at 25°C with a goat antirabbit precipitating complex to separate bound from free tracer. After centrifugation at 760 \times g, the supernatant was aspirated and the bound radioactivity was quantitated in a gamma counter. Human ACTH was used as the standard. Assay sensitivity was approximately 10 pg/ml. The intraassay

coefficient of variation was 3.4% at 41 pg/ml and the inter-assay coefficient of variation was 8.1%.

CRF Receptor Assays

Following the method previously described by De Souza (11), anterior pituitaries and brain regions were thawed, homogenized in 30 vol of cold buffer (50 mM Tris HCl, 10 mM MgCl₂, 2 mM EGTA, pH 7.0 at 22°C) using a Brinkman polytron (setting 5 for 20 s), and centrifuged at 38,000 × *g* for 30 min at 4°C. The resulting pellets were suspended in buffer, washed one more time, and resuspended to a final concentration of 20–40 mg original wet weight of tissue/ml in the same buffer. One hundred microliters of the membrane preparation were added to 1.5 ml polypropylene microtubes containing 100 μl [¹²⁵I-Tyr⁰]-ovine CRF solution (sa = 2200 Ci/mmol; New England Nuclear) ([¹²⁵I]oCRF) and 100 μl incubation buffer [50 mM Tris HCl, 10 mM MgCl₂, 2 mM EGTA, 0.1% BSA, 0.1 mM bacitracin, and aprotinin (100 KIU/ml), pH 7.0 at 22°C]. Half the tubes contained 1 μM rat/human CRF (Peninsula Laboratories, San Carlos, CA) to define nonspecific binding. The reaction was allowed to proceed for 2 h at room temperature. All measurements were made in triplicate. The tissue was separated from the incubation medium by centrifugation in a Beckman microfuge (Beckman Instruments, Fullerton, CA) for 3 min at 12,000 × *g*. The resulting pellet was washed with 1 ml ice-cold phosphate-buffered saline containing 0.01% Triton X-100, and the contents were recentrifuged for 3 min at 12,000 × *g*. The supernatant was aspirated and the radioactivity of the pellet was measured in a gamma counter.

The amount of 0.05–0.1 nM [¹²⁵I]oCRF bound to membrane preparations of various brain regions was measured in samples from individual animals. Single-point determinations of 0.05–0.1 nM [¹²⁵I]oCRF binding to anterior pituitary membrane preparations were performed on anterior pituitaries combined from pairs of animals. For each brain region [¹²⁵I]oCRF binding to membranes from stressed animals and their matched controls were always determined in the same assay. Single concentration measurements of [¹²⁵I]oCRF binding for different brain regions varied from 0.05 to 0.1 nM. [¹²⁵I]oCRF binding to tissues from the two different stress groups (3 and 14 days) were not measured at the same time. Saturation binding data was measured at 0.05–1.0 nM [¹²⁵I]oCRF in anterior pituitary membrane samples pooled from four individual animals per condition per behavioral experiment (four separate replicates of the behavioral experiment were conducted); total and nonspecific binding were measured in duplicate at five concentrations of [¹²⁵I]oCRF. For this assay procedure, using anterior pituitary membranes, the total amount of [¹²⁵I]oCRF bound is typically 10–15% of the concentration added and nonspecific binding is 10–20% of the amount bound. For greater methodological detail, see the original publication of this assay procedure (11). Protein concentrations were determined according to the method of Lowry et al. (31).

Statistics

Statistical evaluations of the significance of differences between mean values from control vs. stressed rats were made by *t*-tests. Estimates for the dissociation constant (*K_d*) and the maximum concentration (*B_{max}*) of [¹²⁵I]oCRF binding sites in membrane preparations from the anterior pituitary gland were determined by using the nonlinear curve-fitting routine MLAB (30) on a DEC system 10 computer. The results were analyzed

TABLE 1

EFFECTS OF 3 DAYS OF CHRONIC STRESS ON PLASMA CORTICOSTERONE AND ACTH CONCENTRATIONS

Plasma Hormone	Control Rats	(<i>n</i>)	Stressed Rats	(<i>n</i>)
Corticosterone				
(μg/dl)	2.44 ± 0.67*	(12)	4.53 ± 0.78†‡	(12)
ACTH (pg/ml)	28.8 ± 3.8	(12)	30.3 ± 6.5	(12)

Values are means ± SEM.

*Deviant outlying values were identified by a range test (6) and data were winsorized, *g* = 1 (50).

†Deviant outlying values were identified by a range test (6) and data were winsorized, *g* = 2 (50).

‡Significantly greater than matched control, *t*-test, one tailed, *p* < 0.05.

using nontransformed data from the saturation curves. Comparison of goodness of fit between rival receptor models was made by applying the *F*-statistic to the difference between the residual sum of squares of the alternative models (17). Although fully reliable significance levels and statistical methods have not been developed for the comparison of nonlinear parameters, good comparisons can be made of the statistical significance of an increased goodness of fit between alternative models by applying the *F*-statistic (33,37).

RESULTS

Plasma ACTH did not differ between control animals and those stressed for 3 days. Plasma corticosterone levels were significantly higher in rats stressed for 3 days than in matched controls (Table 1). As noted in the Method section, care was taken to ensure that all animals were decapitated within a narrow time window (0900–1100 h) to minimize the effects of circadian variation on plasma hormone levels. Note that these animals are sacrificed at the nadir for the daily cycle in plasma corticosterone levels (27). The values we present here in Tables 1 and 2 are consistent with plasma hormone data presented by us and by other investigators for rats exposed to chronic stress. The extremely high plasma ACTH and corticosterone concentrations characteristic of acute stress are not maintained during prolonged stress (44) or long-term stress (26).

There was a significant decrease in the apparent number of pituitary CRF receptors in rats exposed to sustained stress for 3 days (Fig. 1). A model of a single binding site is the best fit to the data from both control and stressed rats. The data from stressed rats are explained best by a model in which the number of binding sites (i.e., *B_{max}*) is reduced but the affinity (i.e.,

TABLE 2

EFFECTS OF 14 DAYS OF CHRONIC STRESS ON PLASMA CORTICOSTERONE AND ACTH CONCENTRATIONS

Plasma Hormone	Control Rats	(<i>n</i>)	Stressed Rats	(<i>n</i>)
Corticosterone				
(μg/dl)	2.20 ± 0.19	(16)	3.39 ± 0.55*	(15)
ACTH (pg/ml)	65.9 ± 22.5	(16)	62.2 ± 12.7	(15)

Values are means ± SEM.

*Significantly greater than matched controls, *t*-test, one tailed, *p* < 0.05.

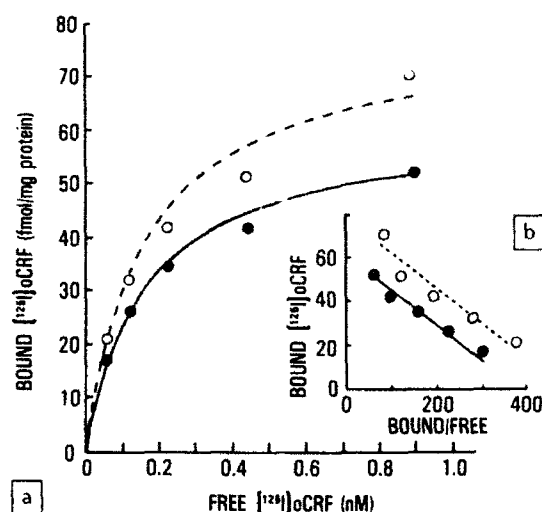


FIG. 1. (a) Effects of chronic stress (3 days) on corticotropin-releasing factor (CRF) receptors in the rat anterior pituitary. Binding of 0.05–1.0 nM [125 I]JoCRF to rat anterior pituitary membranes. Data from controls are open symbols and data from stressed rats are solid symbols. The data are described best by a single-site receptor model of ligand binding, represented by the broken and solid lines for the control and stressed samples, respectively. These data are the mean values from four separate determinations (using pooled pituitaries from four rats per experiment per treatment group). In the generation of parameters of the model giving a least squares-fit solution, both mean amount bound and the variance around the mean from the four separate determinations was used. (b) Eadie-Hofstee plot of data in (a). The decrease of [125 I]JoCRF binding in rats stressed for 3 days is explained best and most simply as a downregulation in the apparent number of binding sites (i.e., decrease in B_{max}). The decrease in [125 I]JoCRF binding to the anterior pituitary membranes from rats stressed for 3 days was consistent in each of the four separate saturation binding experiments.

K_d of the receptor for the ligand is unchanged [for control, $K_d = 0.16 \pm 0.03$ nM, $B_{max} = 78 \pm 24$ fmol/mg protein; for stressed, $K_d = 0.16 \pm 0.02$ nM, $B_{max} = 61 \pm 3$ fmol/mg protein, $F(1, 2) = 109$, $p < 0.01$]. The binding of 0.05–0.1 nM [125 I]JoCRF to membranes from the frontal cortex of rats stressed for 3 days was significantly lower than that in controls (Table 3). There were no other differences in CRF receptors between 3-day stressed and control rats in membrane preparations from the various brain regions examined.

Plasma ACTH did not differ between control animals and those stressed for 14 days. Plasma corticosterone levels were significantly higher in rats stressed for 14 days than in matched controls (Table 2). The plasma hormone concentrations presented here are within the range of values reported by other investigators. In contrast to the data from rats stressed for 3 days, the binding of 0.05–0.1 nM [125 I]JoCRF to anterior pituitary CRF receptors was comparable in membranes from 14-day-stressed rats and control rats (Table 4). [125 I]JoCRF binding was significantly lower in the hypothalamus of rats stressed for 14 days vs. control rats, but binding in the frontal cortex and all other brain regions examined was not different from control values.

Note that the data presented in Tables 3 and 4 do not lend themselves to a comparison of [125 I]JoCRF among various brain regions because the concentration of [125 I]JoCRF varied

TABLE 3
EFFECTS OF 3 DAYS OF CHRONIC STRESS ON 0.05–0.1 nM [125 I]JoCRF BINDING TO MEMBRANES FROM SELECTED BRAIN REGIONS

Tissue	Control	Stressed
Olfactory bulbs	7.67 \pm 0.21	7.79 \pm 0.15
Frontal cortex	6.00 \pm 0.16	5.48 \pm 0.18*
Motor cortex	4.23 \pm 0.21	3.96 \pm 0.11
Somatosensory cortex	6.74 \pm 0.21	6.56 \pm 0.11
Mesolimbic area	3.50 \pm 0.09	3.34 \pm 0.13
Caudate putamen	1.56 \pm 0.07	1.57 \pm 0.06
Hypothalamus	1.61 \pm 0.04	1.58 \pm 0.10
Midbrain†	1.28 \pm 0.05	1.34 \pm 0.06
Cerebellum†	5.13 \pm 0.08	4.86 \pm 0.16

The amount of specifically bound [125 I]JoCRF is in fmol/mg protein. Values are means \pm SEM for $n = 12$ determinations per treatment group.

*Significantly less than control mean, t -test, one tailed, $p < 0.05$.

†Control, $n = 12$; stressed, $n = 11$.

from 0.05 to 0.1 nM in the dozens of separate assays required for these measurements. Regional variation in [125 I]JoCRF binding data using this methodology can be found in DeSouza (11). Likewise, comparison of binding data for any brain region for 3-day-stressed rats (Table 3) vs. 14-day-stressed rats (Table 4) are not appropriate because of varying ligand concentrations as described above and in the Method section. The appropriate comparisons to be made using the data in Tables 3 and 4 are evaluations of any differences between the binding

TABLE 4
EFFECTS OF 14 DAYS OF CHRONIC STRESS ON 0.05–0.1 nM [125 I]JoCRF BINDING TO MEMBRANES FROM ANTERIOR PITUITARY AND SELECTED BRAIN REGIONS

Tissue	Control	Stressed
Anterior pituitary*	24.94 \pm 2.18	24.09 \pm 2.72
Olfactory bulbs	14.52 \pm 0.34	14.31 \pm 0.50
Frontal cortex	8.77 \pm 0.26	8.50 \pm 0.29
Motor cortex	5.77 \pm 0.16	5.94 \pm 0.13
Somatosensory cortex	6.04 \pm 0.11	6.26 \pm 0.20
Mesolimbic area	7.36 \pm 0.23	7.06 \pm 0.19
Caudate putamen	3.41 \pm 0.18	3.21 \pm 0.07
Hypothalamus†	2.63 \pm 0.11	2.31 \pm 0.12‡
Midbrain	1.53 \pm 0.04	1.44 \pm 0.04
Cerebellum	6.52 \pm 0.21	6.38 \pm 0.17

The amount of specifically bound [125 I]JoCRF is in fmol/mg protein. Values are means \pm SEM for $n = 8$ determinations per treatment group for most brain regions.

*Pituitary means are from six separate determinations (pituitaries pooled from two rats per treatment group per three experiments).

†Hypothalamus means are from 16 separate determinations per group.

‡Significantly less than control mean, t -test, one tailed, $p < 0.05$.

of [125 I]oCRF to membrane preparations from experimental animals vs. their matched controls.

DISCUSSION

Stress is known to activate hypothalamic CRF pathways that are involved in regulation of the pituitary hormonal responses to stress (18,40) and is thought to activate extrahypothalamic CRF pathways that have been implicated in coordinating the autonomic and behavioral responses to stress (7,9,46). The present study was conducted to determine whether chronic stress would affect brain and/or pituitary CRF receptors. We utilized a model of sustained stress, characterized in our laboratory (1,2,26), in which rats were exposed to stress of 3 or 14 days' duration. The results of the present study demonstrate that chronic stress of 3 days' duration resulted in increases in the plasma concentrations of corticosterone and decreased anterior pituitary CRF receptors by 22%. In addition, 3 days of chronic stress produced smaller but significant decreases (approximately 10%) in [125 I]oCRF binding in the frontal cerebral cortex. Following 14 days of chronic stress, the number of CRF receptors in the anterior pituitary and frontal cortex of stressed rats was similar to that of controls. However, hypothalamic [125 I]oCRF binding was decreased by approximately 10% in 14-day-stressed rats.

The mechanisms responsible for the observed downregulation of CRF receptors in the brain and anterior pituitary following application of stress are unclear. Chronic stress has been shown to increase both the number and size of CRF-containing neurons in the paraventricular nucleus of the hypothalamus (46), increase the concentration of CRF-like immunoreactivity in the periventricular hypothalamic nucleus and anterior hypothalamic nucleus (9), decrease the concentration of CRF-like immunoreactivity in the median eminence of the hypothalamus (9), and stimulate the secretion of proopiomelanocortin-derived peptides from the corticotrophs of the anterior pituitary (18,35). The decrease in CRF receptor density in the anterior pituitary following 3 days of stress may result from downregulation of CRF receptors by increased endogenous CRF. A similar downregulation of CRF receptors in the anterior pituitary has been demonstrated following adrenalectomy in rats, a procedure that results in increases in the concentration of endogenous CRF (11,22,45,52). In addition, Wynn et al. (51) demonstrated decreased CRF receptors in anterior pituitaries from rats injected intermittently with CRF for a 3-day period. Alternatively, the downregulation of CRF receptors in the anterior pituitary may be a consequence of increased glucocorticoid production following chronic application of stress. In support of the latter hypothesis, Hauger et al. (21) reported that daily administration of 0.5 and 1.0 mg corticosterone for 4 days significantly decreased the relative density of CRF receptors in corticotrophs in culture by increasing the internalization of the receptor.

The decreases in [125 I]oCRF binding in the frontal cortex following 3 days of stress and in the hypothalamus after 14 days of stress are probably in response to stress activation of CRF-containing neurons in these areas. The receptor changes in brain are not likely to be caused by altered corticosterone levels because neither corticosterone administration (21) nor adrenalectomy (10,12,21,52) have been found to affect central CRF receptors. It is not clear what physiological role this small downregulation of CRF receptors may play. It should be kept in mind that the changes we have seen are in relatively large areas of brain tissue composed of several different path-

ways, nuclei, and cell types. The results from this study indicate that we should look at brain CRF receptors in smaller areas of the brain. Examination at the microscopic level may demonstrate larger receptor changes within smaller brain areas.

In contrast to the downregulation of CRF receptors in the anterior pituitary of rats stressed 3 days, normal levels of anterior pituitary CRF receptors were demonstrated in rats stressed for 14 days. The transient nature of this downregulation may reflect successful adaptation of the organism to this chronically stressful milieu. Data presented here and in other reports (26,44) provide evidence for an adaptation of the organism to the hyperactivation of the hypothalamic-pituitary-adrenal (HPA) axis characteristic of acute and short-term stress to a new physiological equilibrium during prolonged, long-term, or chronic stress. Rivier and Vale (44) suggested that reduced pituitary responsiveness to CRF may be a major modulator of the normalization of plasma ACTH levels during prolonged periods of stress. The variety and intensity of the stressor, constant or intermittent nature of the stress, and length of the stress period are all critical variables in the response of the HPA axis to chronic stress. Accordingly, not all studies on chronic stress have demonstrated apparent normalization of the response of the HPA axis during prolonged periods of stress. Clarification of mechanisms of adaptation of the organism to chronic stress may be aided by additional experimentation in which more frequent measurements of relevant indicators of the state of the HPA axis are made during the chronic stress period.

Several lines of evidence suggest that hypersecretion of CRF may be one of a variety of interacting mechanisms associated with abnormalities of the HPA axis in several neuropsychiatric disorders including major depression. Specifically, the concentration of CRF-like immunoreactivity is increased in the cerebrospinal fluid in depressed patients (3,39,49), and the concentration of CRF receptors is significantly decreased (approximately 20%) in the frontal cortex of postmortem tissue obtained from depressed patients (38). Further, the attenuation of the ACTH response to IV administration of CRF in depressed patients when compared to normal controls (19) may reflect partial action or decreased numbers of CRF receptors in the anterior pituitary.

In addition to decreased CRF receptors, there are numerous other physiological perturbations that suggest that this 3-day chronic stress regimen may be a useful animal model in which to study some of the pathologic manifestations of depression. Rats used in this chronic stress paradigm have increased plasma corticosterone, normal ACTH levels, decreased food intake, and increased adrenal weights (1,2,26); similar alterations have been reported in depressed patients (19,20,47). Data demonstrating that ICV administration of CRF decreases food intake (18) and produces behavioral effects characteristic of a "stress" neurotransmitter (4,18) are consistent with CRF hypersecretion in chronic stress, which may result in the downregulation of CRF receptors. Our data demonstrating normal levels of CRF receptors in the anterior pituitary of rats stressed for 14 days may be an indication of successful adaptation to chronic stress. Detailed longitudinal studies of the nature of the physiological alterations of hormones and secretagogues at various times during long-term stress may elucidate some of the mechanisms associated with successful adaptation to stress, failure of which may be contributory to depressive illness.

In summary, we demonstrated that chronic application of

a stressor for 3 days results in alterations in CRF receptors (downregulation of [125 I]oCRF binding in the frontal cortex and decreased density of CRF receptors in the anterior pituitary) and produces other endocrine and behavioral effects similar to those seen in depression. These data provide additional evidence that CRF is a stress neurotransmitter in the CNS and support the role of this peptide in neuropsychiatric disorders. Additional studies are required to elucidate the pre-

cise mechanisms responsible for the alterations in CRF receptors noted in the present study.

ACKNOWLEDGEMENTS

The authors appreciate the excellent technical support provided by Denise Pearsall, Tina Smith, Paul D'Angelo, Terence Eggleston, Golden Driver, Clyde Kenion, Lael Lambe, SGT Gerri Fields, and Clint Wormley.

REFERENCES

- Anderson, S. M.; Leu, J. R.; Kant, G. J. Effects of stress on [3 H]cyclohexyladenosine binding to rat brain membranes. *Pharmacol. Biochem. Behav.* 26:829-833; 1987.
- Anderson, S. M.; Leu, J. R.; Kant, G. J. Chronic stress increases the binding of the A₁ adenosine receptor agonist, [3 H]cyclohexyladenosine to rat hypothalamus. *Pharmacol. Biochem. Behav.* 30:169-175; 1988.
- Banki, C. M.; Bissette, G.; Arato, M.; O'Connor, L.; Nemeroff, C. B. CSF corticotropin-releasing factor-like immunoreactivity in depression and schizophrenia. *Am. J. Psychiatry* 144:873-877; 1987.
- Berridge, C. W.; Dunn, A. J. A corticotropin-releasing factor antagonist reverses the stress-induced changes of exploratory behavior in mice. *Horm. Behav.* 21:393-401; 1987.
- Bissette, G.; Reynolds, G. P.; Kilts, C. D.; Widerlov, E.; Nemeroff, C. B. Corticotropin-releasing factor-like immunoreactivity in senile dementia of the Alzheimer type. *JAMA* 254:3067-3069; 1985.
- Bliss, C. I.; Cochran, W. G.; Tukey, J. W. A rejection criterion based upon the range. *Biometrika* 43:418-422; 1956.
- Britton, K. T.; Lee, G.; Dana, R.; Risch, S. C.; Koob, G. F. Activating and 'anxiogenic' effects of corticotropin releasing factor are not inhibited by blockade of the pituitary-adrenal system with dexamethasone. *Life Sci.* 39:1281-1286; 1986.
- Brodish, A. Control of ACTH secretion by corticotropin-releasing factor(s). *Vitam. Horm.* 37:111-153; 1979.
- Chappell, P. B.; Smith, M. A.; Kilts, C. D.; Bissette, G.; Ritchie, J.; Anderson, C.; Nemeroff, C. B. Alterations in corticotropin-releasing factor-like immunoreactivity in discrete rat brain regions after acute and chronic stress. *J. Neurosci.* 6:2908-2914; 1986.
- Chen, F. M.; Bilezikjian, L. M.; Perrin, M. H.; Rivier, J.; Vale, W. Corticotropin releasing factor receptor-mediated stimulation of adenylate cyclase activity in rat brain. *Brain Res.* 381:49-57; 1986.
- De Souza, E. B. Corticotropin-releasing factor receptors in the rat central nervous system: Characterization and regional distribution. *J. Neurosci.* 7:88-100; 1987.
- De Souza, E. B.; Insel, T. R.; Perrin, M. H.; Rivier, J.; Vale, W. W.; Kuhar, M. J. Differential regulation of corticotropin-releasing factor receptors in anterior and intermediate lobes of pituitary and brain following adrenalectomy in rats. *Neurosci. Lett.* 56:121-128; 1985.
- De Souza, E. B.; Perrin, M. H.; Insel, T. R.; Rivier, J.; Vale, W. W.; Kuhar, M. J. Corticotropin-releasing factor receptors in rat forebrain: Autoradiographic identification. *Science* 224:1449-1451; 1984.
- De Souza, E. B.; Whitehouse, P. J.; Folstein, S. E.; Price, D. L.; Vale, W. W. Corticotropin-releasing hormone (CRH) is decreased in the basal ganglia in Huntington's disease. *Brain Res.* 437:355-359; 1987.
- De Souza, E. B.; Whitehouse, P. J.; Kuhar, M. J.; Price, D. L.; Vale, W. W. Reciprocal changes in corticotropin-releasing-factor (CRF)-like immunoreactivity and CRF receptors in cerebral cortex of Alzheimer's disease. *Nature* 319:593-595; 1986.
- De Souza, E. B.; Whitehouse, P. J.; Kuhar, M. J.; Price, D. L.; Vale, W. W. Abnormalities in corticotropin-releasing hormone (CRH) in Alzheimer's disease and other human disorders. *Ann. NY Acad. Sci.* 512:237-247; 1987.
- Draper, N. R.; Smith, H. Applied regression analysis. New York: John Wiley & Sons; 1981:97-101.
- Emeric-Sauval, E. Corticotropin-releasing factor (CRF)—a review. *Psychoneuroendocrinology* 11:277-294; 1986.
- Gold, P. W.; Calabrese, J. R.; Kling, M. A.; Augerinos, P.; Khan, I.; Gallucci, W. T.; Tomai, T. P.; Chrousos, G. P. Abnormal ACTH and cortisol responses to ovine corticotropin releasing factor in patients with primary affective disorder. *Prog. Neuro-psychopharmacol. Biol. Psychiatry* 10:57-65; 1986.
- Gold, P. W.; Chrousos, G.; Kellner, C.; Post, R.; Roy, A.; Augerinos, P.; Schulte, H.; Oldfield, E.; Loriaux, D. L. Psychiatric implications of basic and clinical studies with corticotropin-releasing factor. *Am. J. Psychiatry* 141:619-627; 1984.
- Hauger, R. L.; Millan, M. A.; Catt, K. J.; Aguilera, G. Differential regulation of brain and pituitary corticotropin-releasing factor receptors by corticosterone. *Endocrinology* 120:1527-1533; 1987.
- Holmes, M. C.; Catt, K. J.; Aguilera, G. Involvement of vasopressin in the downregulation of pituitary corticotropin-releasing factor receptors after adrenalectomy. *Endocrinology* 121:2093-2098; 1987.
- Hotta, M.; Shibasaki, T.; Masuda, A.; Imaki, T.; Demura, H.; Ling, N.; Shizume, K. The responses of plasma adrenocorticotropin and cortisol to corticotropin-releasing hormone (CRH) and cerebrospinal fluid immunoreactive CRH in anorexia nervosa patients. *J. Clin. Endocrinol. Metab.* 62:319-324; 1986.
- Jacobowitz, D. M. Removal of discrete fresh regions of the rat brain. *Brain Res.* 80:111-115; 1974.
- Kant, G. J.; Anderson, S. M.; Dhillon, G. S.; Mougey, E. H. Neuroendocrine correlates of sustained stress: The activity stress paradigm. *Brain Res.* 20:407-414; 1988.
- Kant, G. J.; Leu, J. R.; Anderson, S. M.; Mougey, E. H. Effects of chronic stress on plasma corticosterone, ACTH and prolactin. *Physiol. Behav.* 40:775-779; 1987.
- Kant, G. J.; Mougey, E. H.; Meyerhoff, J. L. Diurnal variation in neuroendocrine response to stress in rats: Plasma ACTH, β -endorphin, β -LPH, corticosterone, prolactin and pituitary cyclic AMP responses. *Neuroendocrinology* 43:383-390; 1986.
- Kant, G. J.; Mougey, E. H.; Pennington, L. L.; Meyerhoff, J. L. Graded foot-shock stress elevates pituitary cyclic AMP and plasma β -endorphin, β -LPH, corticosterone and prolactin. *Life Sci.* 33:2657-2663; 1983.
- Klimek, A.; Cieslak, D.; Szulc-Kuberska, J.; Stepień, H. Reduced lumbar cerebrospinal fluid corticotropin releasing factor (CRF) levels in amyotrophic lateral sclerosis. *Acta Neurol. Scand.* 74:72-74; 1986.
- Knott, G. D. MLAB—a mathematical modeling tool. *Comp. Programs Biomed.* 10:271-280; 1979.
- Lowry, O. H.; Rosenbrough, N. A.; Farr, A. L.; Randall, R. J. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193:265-275; 1951.
- Lowry, P. J.; Estivariz, F. E.; Gillies, G. E.; Kruseman, A. C. N.; Linton, E. A. CRF: Its regulation of ACTH and pro-opiomelanocortin peptide release and its extra hypothalamic occurrence. *Acta Endocrinol.* 276(suppl.):56-62; 1986.
- Mannervik, B. Regression analysis, experimental error, and statistical criteria in the design and analysis of experiments for discrimination between rival kinetic models. In: Purich D. L., ed. *Methods in enzymology*. vol. 87. Enzyme kinetics and mechanisms. New York: Academic Press; 1982:370-390.
- Merchenthaler, I.; Vigh, S.; Petrusz, P.; Schally, A. V. The paraventricular-infundibular corticotropin-releasing factor (CRF)

- pathway as revealed by immunocytochemistry in long-term hypophysectomized or adrenalectomized rats. *Reg. Peptides* 5:295-306; 1983.
35. Meyerhoff, J. L.; Mougey, E. H.; Kant, G. J. Paraventricular lesions abolish the stress-induced rise in pituitary cyclic adenosine monophosphate and attenuate the increases in plasma levels of proopiomelanocortin-derived peptides and prolactin. *Neuroendocrinology* 46:222-230; 1987.
 36. Mougey, E. H. A radioimmunoassay for tetrahydrocortisol. *Anal. Biochem.* 91:566-582; 1978.
 37. Munson, P. J.; Rodbard, D. LIGAND: A versatile computerized approach for characterization of ligand-binding systems. *Anal. Biochem.* 107:220-239; 1980.
 38. Nemeroff, C. B.; Owens, M. J.; Bissette, G.; Andorn, A. C.; Stanley, M. Reduced corticotropin releasing factor binding sites in the frontal cortex of suicide victims. *Arch. Gen. Psychiatry* 45: 577-579; 1988.
 39. Nemeroff, C. B.; Widerlov, E.; Bissette, G.; Walleus, H.; Karlsson, I.; Eklund, K.; Kilts, C. D.; Loosen, P. T.; Vale, W. Elevated concentration of CSF corticotropin-releasing factor-like immunoreactivity in depressed patients. *Science* 226:1342-1344; 1984.
 40. Ono, N.; Samson, W. K.; McDonald, J. K.; Lumpkin, M. D.; Bedran de Castro, J. C.; McCann, S. M. Effects of intravenous and intraventricular injection of antisera directed against corticotropin-releasing factor on the secretion of anterior pituitary hormones. *Proc. Natl. Acad. Sci. USA* 82:7787-7790; 1985.
 41. Palkovits, M.; Brownstein, M. J.; Vale, W. Distribution of corticotropin-releasing factor in rat brain. *Fed. Proc.* 44:215-219; 1985.
 42. Paxinos, G.; Watson, C. The rat brain in stereotaxic coordinates. New York: Academic Press; 1982.
 43. Reisine, T.; Hoffman, A. Desensitization of corticotropin-releasing factor receptors. *Biochem. Biophys. Res. Comm.* 111: 919-925; 1983.
 44. Rivier, C.; Vale, W. Diminished responsiveness of the hypothalamic-pituitary-adrenal axis of the rat during exposure to prolonged stress: A pituitary-mediated mechanism. *Endocrinology* 121:1320-1328; 1987.
 45. Rivier, C. L.; Plotsky, P. M. Mediation by corticotropin releasing factor (CRF) of adeno-hypophysial hormone secretion. *Annu. Rev. Physiol.* 48:475-494; 1986.
 46. Silverman, A.-J.; Hou-Yu, A.; Kelly, D. D. Modification of hypothalamic neurons by behavioral stress. In: Tache, Y.; Morley, J. E.; Brown, M. R., eds. *Hans Selye Symposium: Neuropeptides and stress*. New York: Springer-Verlag; 1989:23-38.
 47. Spitzer, R. L.; Endicott, J.; Robins, E. Research diagnostic criteria, rational and reliability. *Arch. Gen. Psychiatry* 35:773-782; 1978.
 48. Swanson, L. W.; Sawchenko, P. E.; Rivier, J.; Vale, W. W. Organization of ovine corticotropin-releasing factor immunoreactive cells and fibers in the rat brain: An immunohistochemical study. *Neuroendocrinology* 36:165-186; 1983.
 49. Widerlov, E.; Bissette, G.; Nemeroff, C. B. Monoamine metabolites, corticotropin releasing factor and somatostatin as CSF markers in depressed patients. *J. Affect. Disorders* 14:99-107; 1988.
 50. Winer, B. J. Statistical principles in experimental design. ed. 2. New York: McGraw-Hill; 1971:51-54.
 51. Wynn, P. C.; Harwood, J. P.; Catt, K. J.; Aguilera, G. Corticotropin-releasing factor (CRF) induces desensitization of the rat pituitary CRF receptor-adenylate cyclase complex. *Endocrinology* 122:351-358; 1988.
 52. Wynn, P. C.; Hauger, R. L.; Holmes, M. C.; Millan, M. A.; Catt, K. J.; Aguilera, G. Brain and pituitary receptors for corticotropin releasing factor: Localization and differential regulation after adrenalectomy. *Peptides* 5:1077-1084; 1984.

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